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Assay for Influenza Virus Endonuclease Using DNA Polymerase Extension of a Specific Cleavage Product

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The synthesis of influenza virus mRNA requires primers generated by cleavage of host cell transcripts 10–13 nucleotides from the 5′ end by a virally encoded endonuclease. This novel enzyme is an attractive target for the development of antiviral agents. An assay for the influenza virus endonuclease has been developed that monitors the substrate cleavage reaction only at the correct position in the sequence, thereby discriminating against nonspecific RNA cleavage products. The influenza endonuclease assay is sensitive enough to detect 200 amol of product. The assay employs a DNA polymerase-catalyzed extension of the endonuclease cleavage product using radiolabeled dGTP and a DNA template containing a 3′ region complementary to the product joined to a 5′ region consisting of 10 dC residues. The influenza endonuclease assay does not involve gel electrophoretic separation and is amenable to high volume screening of potential inhibitors. The assay may also be employed to determine the site of influenza endonucleolytic cleavage in the substrate.

The influenza A virus genome consists of eight negative-sense single-stranded RNA segments. Synthesis of viral mRNA is catalyzed by a viral-encoded RNA-dependent RNA polymerase (EC 2.7.7.48) consisting of three polypeptides: PA, PB1, and PB2, which are complexed at the 3′ end of each segment. Influenza virus transcription is initiated by a novel mechanism in which primers are generated by cleaving host cell transcripts 10–13 nt[2] from their 5′ cap structure (1). The cleavage and priming reactions are dependent on the transcript possessing a cap-1 structure containing a 7-methylated terminal G and a 2′-O-methylated penultimate purine base (m^7GpppRm). The PB2 protein recognizes and binds to the cap-1 structure and the PB1 protein catalyzes the polymerization reactions (2,3). The identity of the endonuclease protein within this complex is not known. No specific function has been assigned to the PA protein in the cap-1-dependent transcription reactions.

Influenza transcription represents an attractive target for development of antiviral agents. The P proteins are highly conserved between influenza A and B. The PB1 protein exhibits 60% homology between these types, which is the highest homology observed for any influenza protein (4,5). We are particularly interested in discovering specific inhibitors of the cap-dependent endonuclease activity of the influenza transcriptase complex because this activity is not present in the host cell. Small molecule (6) and oligonucleotide (7) inhibitors of the influenza endonuclease have recently been described.

Screening for potential inhibitors of the influenza endonuclease has been hampered by the lack of a suitable assay method. The requirements for such an assay are: (a) high throughput, (b) the ability to distinguish influenza endonuclease-catalyzed cleavage from nonspecific RNA cleavage, and (c) high sensitivity. Previous endonuclease assays involved the use of polyacrylamide gel electrophoresis to separate product from substrate (8), which is not convenient for processing large numbers of samples. Assays have also been described for the overall influenza transcriptase reaction (9) which may be capable of detecting inhibition of the endonuclease. However, the overall reaction is a complex, multistep process in which cleavage may not be rate-limiting and endonuclease inhibitors may be missed. Therefore, we have developed a novel DNA polymerase extension assay which is specific for the influenza virus endonuclease. This assay does not involve an electrophoresis step and may be run in a 96-

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[2] Abbreviations used: AlMV, alfalfa mosaic virus; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; nt, nucleotide; R, a purine nucleoside; 5× SSC, 0.75 M NaCl, 75 mM sodium citrate, pH 7.0; Tris, tris[hydroxymethyl]aminomethane.

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well microtiter plate format. Other key features of the assay are that it monitors the substrate cleavage reaction only at the correct position in the sequence, thereby discriminating against nonspecific RNA cleavage products, and that it is sensitive enough to detect 200 amol of product.

MATERIALS AND METHODS

Materials

Sequenase Version 2.0 was obtained from United States Biochemicals (Cleveland, OH). Oligodeoxyribonucleotides and uncapped oligoribonucleotides were synthesized by Midland Certified Reagent Company (Midland, TX) and were purified by anion-exchange HPLC. Triphosphorylated oligoribonucleotide substrates were synthesized and capped as previously described (D. B. Olsen, manuscript in preparation). The sequence of the 19-nt substrate oligoribonucleotide is 5′-GUUUUUAUUUUUAAUUUUC-3′. Oligoribonucleotides were also synthesized which correspond to the 5′ region of the substrate with lengths of 14, 13, 12, 10, and 6 nt. Unless otherwise indicated, a 23-nt DNA template was used in all experiments with the sequence 5′-biotin-CCCCCCCCCCTAAAAATAAAAAC-amino-3′, where the 5′-biotin is a *N*-biotinyl-6-aminohexyloxyphosphoryl moiety and the 3′-amino is a (3-amino-2-hydroxy)-propoxyphosphoryl moiety. For some experiments a 24-nt DNA template was used with the sequence 5′-biotin-CCCCCCCCCCTTAAAAATAAAAAC-amino-3′. [α-^{32}P]dGTP (3000 Ci/mmol) was obtained from Dupont NEN. Unlabeled dGTP was obtained from Pharmacia. Viral cores were purified from influenza virus A/PR/8 as described (D. B. Olsen, manuscript in preparation).

Methods

Nucleic acid melting curves were obtained at a concentration of 0.8 μM duplex in 1 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.0. The samples were annealed by heating to 65°C for 1 min and cooled to 4°C over 10 min. The melting curves were recorded on an AVIV 14 uv–vis spectrophotometer equipped with an automated temperature controller.

Influenza cleavage reactions were performed at 25°C in a buffer containing 100 mM Tris–Cl, 50 mM KCl, 0.25 mM $MgCl_2$, 5 mM DTT, 4% (v/v) DMSO, pH 8.0, in diethyl pyrocarbonate-treated water. Sequenase extension reactions were performed in the same buffer, except that the $MgCl_2$ concentration was increased to 10 mM and the DMSO concentration to 9%. Unlabeled dGTP (500 nM) was employed in extension reactions using end-labeled primers and a mixture of 50 nM [α-^{32}P]dGTP and 450 nM unlabeled dGTP was employed with unlabeled primers. Unless otherwise indicated, extension reactions were performed at 0°C.

Reactions were analyzed either by electrophoresis in 20% polyacrylamide gels containing 8 M urea or by filtering through 0.2-μm pore Nytran membranes in a 96-well manifold (Schleicher and Schuell, Keene, NH). Prior to filtration, samples were diluted with 200 μl of 250 mM EDTA, pH 8.0, and 200 μl was loaded onto the membrane equilibrated in 5× SSC and filtered immediately. Each well was washed five times with 200 μl of 5× SSC, and then the filter was removed from the manifold and washed three times in 100 ml of 5× SSC.

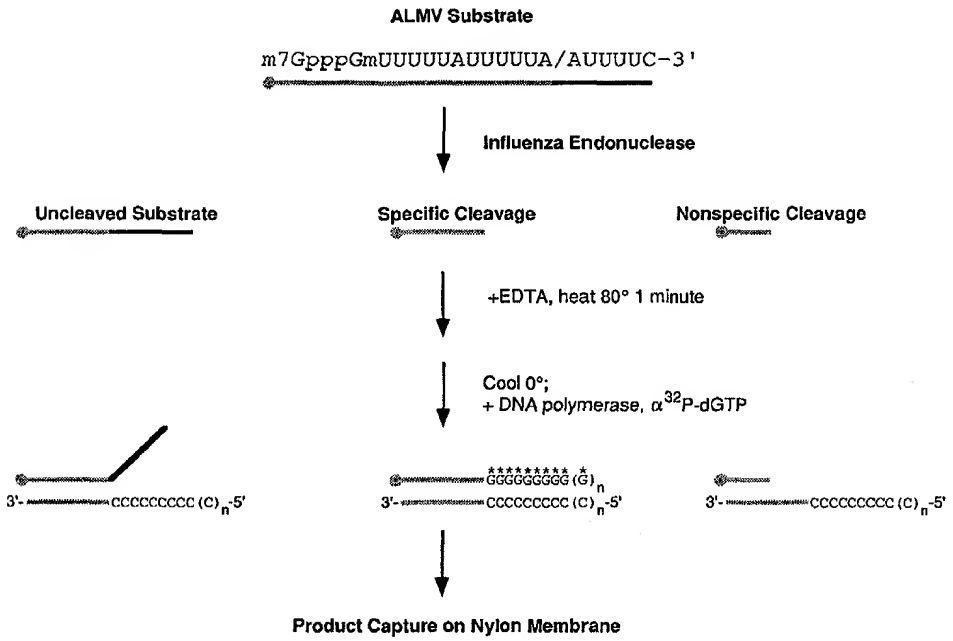
Gels were visualized and filters were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) with the Imagequant software provided by the manufacturer.

RESULTS AND DISCUSSION

Principle of the Assay

The principle of the assay is depicted in Scheme 1. A 19-nt 5′-capped RNA substrate is cleaved by the influenza endonuclease, generating a 13-nt capped product. This product is then used as a primer for a DNA polymerase-catalyzed extension reaction with α-^{32}P-labeled dGTP. The endonuclease substrate sequence is derived from the 5′ end of the AlMV 4 RNA, which has been demonstrated to be a substrate for the influenza endonuclease and is cleaved 3′ to A13 (D. B. Olsen, manuscript in preparation). The DNA template for this reaction contains a 3′ region complementary to the AlMV primer joined to a 5′ region consisting of 10 dC residues. In addition, the 3′-OH is blocked by an aminolinker moiety. Following the cleavage reaction, the 13-nt product hybridizes with the template and it is extended by the addition of 10 α-^{32}P-labeled dG residues catalyzed by a DNA polymerase. We have chosen to use Sequenase Version 2, a mutant of bacteriophage T7 DNA polymerase in which the 3′ → 5′ exonuclease activity was abolished by *in vitro* mutagenesis (10). The useful properties of this enzyme include the ability to use oligoribonucleotides as primers, high fidelity, and the absence of a proofreading activity. Signal detection is achieved by filtering the reaction mixture through a nylon membrane. The unincorporated dGTP flows through the membrane, while the extended primer is retained. The amount of radioactivity bound to the filter is quantitated using a Phosphorimager or by a plate reading scintillation counter.

The specificity of the assay is achieved through the design of the template and the high fidelity of the T7 DNA polymerase. Under the conditions of a typical reaction >90% of the substrate is not cleaved, but the uncleaved RNA substrate does not serve as a primer for polymerization because the 3′ end cannot base pair to the template. In addition, nonspecific cleavage prod-



SCHEME 1. Principle of the DNA polymerase extension assay for influenza virus endonuclease.

ucts with 3′ termini other than A13 will not be extended. Although these shorter sequences may hybridize with the template, they are not extended by the DNA polymerase because this reaction would require dATP or dTTP, which are not supplied (Scheme 1).

Extension Reactions

Figure 1 demonstrates the specificity of the Sequenase-catalyzed extension reaction and the effects of temperature. Synthetic, uncapped RNA and DNA primers of various lengths and sequence were hybridized with the DNA template and extended with Sequenase and [α-^{32}P]dGTP. When the reaction is carried out at 0°C, the only primers that give efficient extension are the 13-nt AlMV RNA and the corresponding sequence in DNA. Although incompletely extended products are visible on the gel, the reaction conditions have been optimized such that the most intense band corresponds to complete primer extension. A fainter band above the most intense band corresponds to a single base addition to the full-length product. Several DNA polymerases are known to catalyze the addition of a single base onto the 3′-OH termini of a blunt-ended DNA (11); presumably, Sequenase exhibits a similar activity. The band in the lane containing the 14-nt RNA primer is less than 25% the intensity of the band in the lane containing the 13-nt RNA primer and is attributable to a contaminant of 13-nt RNA present in the 14-nt ribonucleotide. No extension products are observed using either the full-length 19-nt primer, AlMV primers smaller than 13 nt, or a heterologous 13-nt RNA derived from the 5′ end of the β-globin transcript (5′-ACACUUGCUUUUG-3′). Thus, the Sequenase reaction selectively extends the AlMV 13-nt primers and discriminates against sequences corresponding to uncleaved AlMV substrate or shorter nonspecific cleavage products. It is noteworthy that the high fidelity of

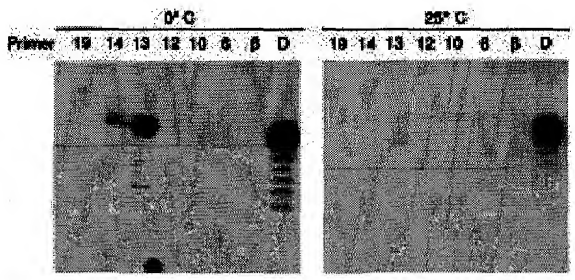


FIG. 1. Effects of primer length and temperature on Sequenase-catalyzed extension reactions. Various primers were hybridized to the 23-nt DNA template and extended with sequenase and dGTP. The lane labeled 19 corresponds to an uncapped RNA primer with the sequence of the AlMV substrate (5′-GUUUUUAUUUUUAAUUUUC-3′); lanes labeled 14–6 correspond to RNA primers of the indicated length derived from 3′ deletions of the 19-nt primer. The lane labeled β corresponds to a 13-nt RNA derived from the 5′ end of β-globin mRNA (5′-ACACUUGCUUUUG-3). The lane labeled D corresponds to a 13-nt DNA with the AlMV primer sequence (5′-GTTTTTATTTTTA). The reactions contained 1 nM primer, 50 nM template, 500 nM Sequenase, and 500 nM dGTP and were carried out for 2 h at the indicated temperature. The reactions were analyzed on 20% polyacrylamide 8 M urea gels. The spot at the bottom of the lane with a 12-nt primer carried out at 0°C is an artifact.

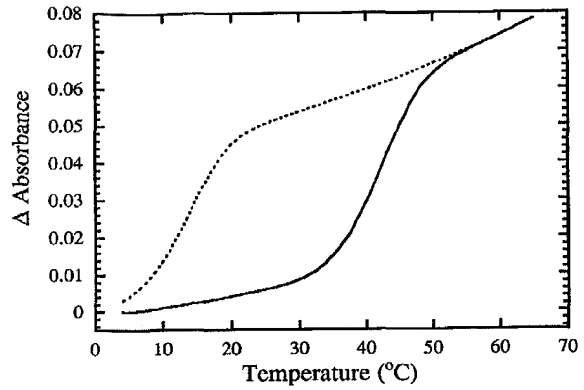


FIG. 2. Melting of AlMV DNA:DNA and DNA:RNA duplexes. (—), DNA:DNA duplex, d(TAAAAATAAAAAC):d(GTTTTTATTTTA); (---), DNA:RNA duplex, d(TAAAAATAAAAAC):r(GUUUUUAUUUUA). The nucleic acid absorbance (260 nm, 4-nm spectral bandwidth) was recorded over a temperature range of 4 to 65°C (DNA:DNA) or 4 to 60°C (RNA:DNA) at 0.5°C intervals with 30 s integration per point.

Sequenase prevents incorrect extension of AlMV primers differing in length by as little as a single base.

Figure 1 shows that if the extension reactions are carried out at 25°C instead of 0°C, the 13-nt AlMV RNA no longer serves as a primer but the reaction with the corresponding DNA sequence is unaffected. In order to determine the origin of this effect of temperature on extension of the RNA primer, melting curves were obtained using 13-nt DNA and RNA oligonucleotides with the AlMV sequence and a 13-nt complementary DNA oligonucleotide. A cooperative transition is observed for both the DNA:DNA and RNA:DNA duplexes (Fig. 2) but the former melts at 42°C, whereas the latter melts at a much lower temperature of 14°C. Thus, the absence of Sequenase extension products when using an RNA primer at 25°C is due to a lack of primer:template hybridization. Earlier studies have demonstrated that the relative stability of DNA:DNA and RNA:DNA duplexes is dependent on sequence (12); in particular, it has been demonstrated that the rU:dA base pair is much less stable than dT:dA (13). The AlMV RNA contains 10 rU residues of a total of 13, so the marked instability of the RNA primer:DNA template duplex is reasonable. For effective hybridization of the RNA primer to the DNA template, all subsequent Sequenase extension reactions are carried out at 0°C, well below the melting temperature of 14°C.

We have found that the Sequenase extension reaction can be conveniently monitored by filtration through nylon membranes. Under the conditions we employ, we observe quantitative binding of the extended product, whereas <0.0003% of the unincorporated [α-^{32}P]dGTP is retained. For high-volume screening purposes the filtration process can be performed using a 96-well manifold and the filter bound radioactivity can be quantitated with either a Phosphorimager or a microplate scintillation counter. Figure 3 shows the sensitivity and linear response using nylon membrane filtration and Phosphorimager detection for extension of the 13-nt AlMV RNA primer. Excellent linearity is observed up to 200 pM primer. Note that under typical assay conditions approximately 10% of the substrate will be converted, corresponding to 40 pM of product; this amount is well within the linearity range and detection limits of the assay.

A background primer-independent reaction is observed, manifested as a faint band running above the major extension products across all lanes in Fig. 1 and as a nonzero y-intercept in Fig. 3. In the absence of template the faint band is not visible and y-intercept is decreased, suggesting that these features are correlated. The background signals are independent of the presence of viral core but they are dependent on Sequenase. Thus, the background is due to a sequenase-catalyzed addition of [α-^{32}P]dGTP to the single-stranded template. Much higher background signals are observed using a template in which the 3′-OH group is not blocked by an aminolinker moiety, suggesting that the reaction requires a free 3′-OH. Recently, we have found that introduction of a 3′-3′-A-5′ linkage at the 3′-end of the template more effectively blocks the 3′-OH than the aminolinker (J. L. Cole, D. B. Olsen, and F. Benseler, unpublished observations). In addition, the relative contribution of the background to the observed signal potentially can be decreased by using templates with longer stretches of dC residues.

In order to clearly characterize the combined influ-

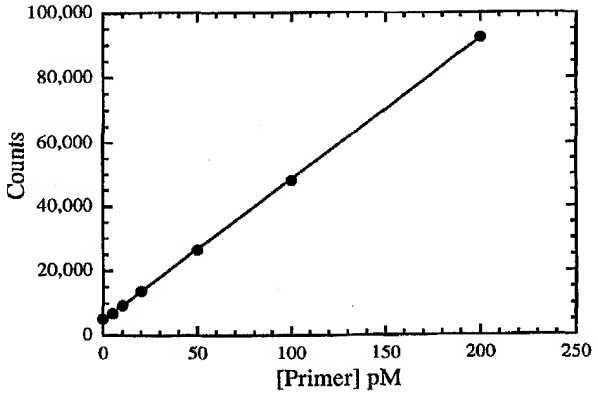


FIG. 3. Linear response of DNA polymerase extension. Each reaction was carried out in a 20-μl volume containing the indicated concentration of the AlMV 13-nt primer, 50 nM template, 50 nM Sequenase, and 500 nM dGTP for 18 h at 0°C. Two-hundred microliters of 250 mM EDTA, pH 8.0 was added and 200 μl of each sample was filtered in a 96-well manifold onto a Nytran membrane. Following washing, the membrane was exposed to a Phosphorimager screen for 1 h and quantitated using Imagequant software. The line drawn through the points is a linear least-squares fit to the data with a correlation coefficient of 0.9999.

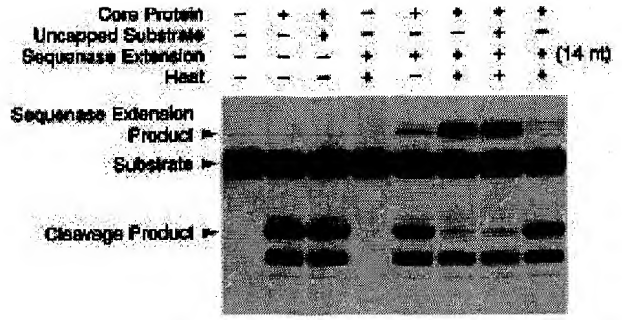


FIG. 4. Sequenase-catalyzed extension of influenza endonuclease cleavage product. The cleavage reaction was carried out using 0.4 nM $m^7G^{32}P$-labeled AlMV substrate and 1 μl viral core in a 15-μl volume at 25°C for 30 min. The reaction was quenched by addition of 0.4 mM EDTA. The extension reaction was carried out in a 20-μl volume containing 500 nM Sequenase, 500 nM dGTP, 50 nM template (13- or 14-nt complementary region). The reaction proceeded for 2 h at 0°C and was quenched by addition of an equal volume of stop mix. Ten microliters was loaded into each well of a 20% acrylamide, 8 M urea gel. The gel was exposed to a Phosphorimager screen for 3 days.

enza endonuclease/Sequenase extension process, a series of reactions were carried out using $m^7G^{32}P$-labeled AlMV substrate and unlabeled dGTP and were analyzed by gel electrophoresis. Figure 4 shows that in the absence of viral cores the AlMV substrate migrates as a single band corresponding to a length of 19 nt. Incubation of substrate with viral cores results in a major cleavage product as well as minor products that are shorter. We have demonstrated that the major product corresponds to cleavage at A13 and that this product is extended upon incubation with GTP (D. B. Olsen, manuscript in preparation) and similar results have been reported using the full-length AlMV RNA 4 (8). Addition of a 10-fold excess of unlabeled uncapped AlMV substrate does not affect the extent of cleavage. Incubation of substrate with Sequenase, template, and dGTP does not result in any extension in the absence of viral cores. Cleavage of substrate by influenza endonuclease followed by Sequenase extension results in the appearance of a faint band above the substrate band. This band corresponds to the addition of 10 dG residues to the 13-nt product. Note that in this sample the bulk of the cleavage product is not extended by sequenase. In contrast, in the sample which is incubated at 80°C for 1 min following cleavage, near-quantitative extension is observed. The dissociation of the cleavage product is likely to be quite slow and the heating step may serve to release the bound product by denaturing the influenza endonuclease complex. A faint band observed above the sequenase extension product likely corresponds to addition of an extra dG residue to the blunt-ended extended primer:template duplex, as was observed in Fig. 1. As in the case of the cleavage reaction alone, the combined cleavage/extension reaction is not affected by the presence of a 10-fold excess of unlabeled uncapped AlMV substrate. In the last lane the 24-nt template that contains a 14-nt complementary region was used instead of the 13-nt complementary template. The specific cleavage product is not extended in this sample, which confirms that the major product corresponds to cleavage at A13 and not at A14.

The DNA polymerase extension assay was validated for detecting influenza endonuclease inhibitors using the inhibitor 4-[*N*-benzenesulfonyl-3-(4-chlorobenzyl)-piperidin-3-yl]-2,4-dioxobutanoic acid, which we have previously identified using the gel-based assay. This compound is similar to the 4-substituted 2,4-dioxobutanoic acids recently described as inhibitors of the influenza endonuclease (6). Figure 5 shows titrations of the inhibition of the endonuclease by this compound using the gel-based assay and the DNA polymerase extension

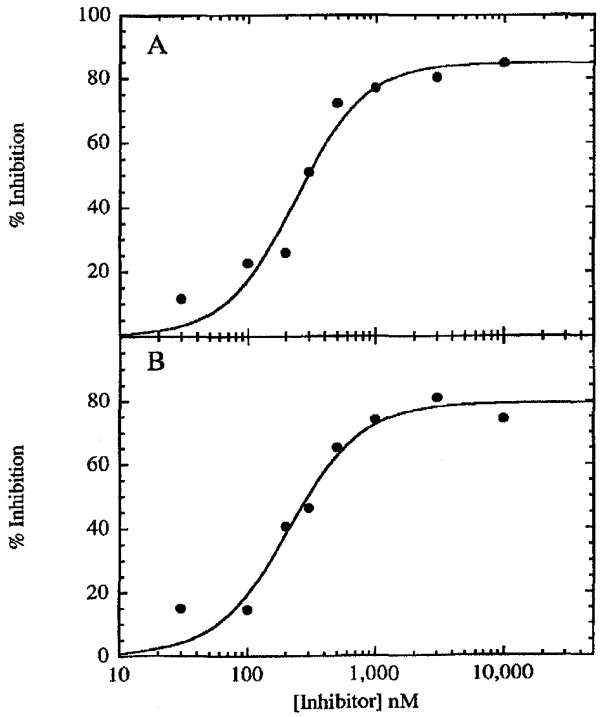


FIG. 5. Comparison of the inhibition of the influenza endonuclease detected with (A) the gel-based assay and (B) the DNA polymerase extension assay. The compound 4-[*N*-benzenesulfonyl-3-(4-chlorobenzyl)piperidin-3-yl]-2,4-dioxobutanoic acid was preincubated with viral cores for 10 min. The cleavage reactions were initiated by adding 0.4 nM of unlabeled (gel-based assay) or endlabeled (DNA polymerase extension assay) substrate in a 15-μl volume reaction containing 0.75-μl viral cores and were run for 10 min at 25°C. For the latter assay, the extension reaction was carried out in a 20-μl volume containing 50 nM Sequenase, 500 nM dGTP, 50 nM template for 18 h at 0°C. The inhibition data were fit to the equation %Inhibition = $100\ A_{max}([L]/IC_{50})^n/(1 + [L]/IC_{50})^n$. For the gel-based assay data, A_{max} = 84, IC_{50} = 238 ± 40 nM, and n = 1.58 ± 0.41 and for the DNA polymerase extension assay, A_{max} = 79, IC_{50} = 211 ± 34 nM, and n = 1.52 ± 0.36.

assay under the same experimental conditions. The IC_{50} values determined by fitting the data are 238 ± 40 nM for the gel-based assay and 211 ± 34 nM for the DNA polymerase extension assay. Within error, the potency of this compound is the same in both assays, indicating that the DNA polymerase extension assay accurately monitors inhibition of the influenza endonuclease.

The DNA polymerase extension assay provides a convenient method to determine the cleavage position of capped substrates. The site of cleavage by the influenza endonuclease has been previously determined by generating sequence ladders with alkaline digestion (8) or by digestion with RNases (7) and electrophoresis. The former method suffers from ambiguities arising from lability of the m7G residue in the cap structure to alkaline hydrolysis and the latter from the faster electrophoretic mobility of the 3′-phosphorylated sequences generated by RNases relative to the unphosphorylated 3′-OH ends generated by the influenza endonuclease. In contrast, the cleavage site can be unambiguously defined using the DNA polymerase extension assay by comparing the extension reactions catalyzed using templates with complementary regions corresponding to the expected cleavage products. Figure 4 provides a clear example of this approach, in which the template with a 13-nt complementary region serves as a substrate, whereas the 14-nt complementary template does not.

The principle of the DNA polymerase extension assay should be applicable to other enzymes that give rise to oligonucleotide products. For example, ribozymes mediate intra- or intermolecular RNA cleavage reactions (14) that could be monitored using appropriately designed templates. Alternatively, transcriptional activation reactions could be monitored by using templates with a complementary region corresponding to the expected transcript. A chief advantage of the DNA polymerase extension assay over existing methodologies is that the oligonucleotide product of interest could be detected with high sensitivity even in the presence of other sequences provided that they do not serve to prime polymerization. Thus, precise measurements of the reaction of interest could be performed even in complex, impure preparations.

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